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Velvet antler polypeptides promoted proliferation of epidermal cells and fibroblasts and skin wound healing

Abstract:

Aim: To study the effects of total velvet antler polypeptides (TVAP), native VAP (nVAP) and synthetic VAP) sVAP) on proliferation of epidermal cells and fibroblasts.

Methods: Newborn rat epidermal cells and rabbit chondrocytes were isolated and cultured in the medium containing TVAP or nVAP and sVAP. Mitogenic effects of these polypeptides were determined by measuring [^{3}H]-TdR incorporation into DNA. Effects of TVAP on Skin wound healing in rats was observed.

Results: Ointment containing 0.8 and 3.2 mg TVAP /g body weight, which was applied externally, accelerated healing of rat back skin wound. The wound-remaining rate of TVAP-treated group was lower (7.08%±4.57%) than that of the control group (16.16%±6.52%) ($p<0.01$) at day 17 after treatment. nVAP at 0.4, 2, 10 and 50 mg/L showed most marked proliferation-promoting activity for rat epidermal cells and increased incorporation of [^{3}H] TdR from (27±10) Bq (control group) to (112±32), (178±43), (271±48) and (323±32) Bq, respectively ($P<0.01$ or $P<0.001$). It showed that nVAP from TVAP is the main active polypeptides responsible for stimulating proliferation of epidermal cells and skin wound healing. sVAP also showed stimulating effects on epidermal cells and fibroblasts.

Conclusion: TVAP accelerated skin wound healing by stimulating proliferation of epidermal cells and fibroblasts.

Key words: velvet antler; velvet antler polypeptides; epidermal cells; fibroblasts; skin wound

Introduction

Based on the biological property of rapid growth, we isolated total polypeptides from wapiti (*Cervus elaphus* Linnaeus) velvet antler. These polypeptides can significantly stimulate proliferation of epidermal cells. Subsequently, we produced new class II drug using the polypeptides: "injectable velvet extracts for wound healing". With the support of Natural Science Funding, we successfully isolated a single compound from the polypeptides, called native velvet antler polypeptide (nVAP) from total polypeptides. nVAP is a peptide containing 32 amino acids, and its MW is 3,216 u, pI=8.15. nVAP mainly contains valine, alanine, lysine and glycine, but does not contain cysteine. The amino acid sequence of nVAP has been confirmed and is less than 50% homology with known polypeptides. Preliminary results showed it is a newly discovered polypeptide from wapiti velvet antler. Based on the sequence of nVAP, recently we synthesized a polypeptide (synthetic velvet antler polypeptide, sVAP). The MW the sVAP is 3 200 u. This paper

reported the effects of total velvet antler polypeptides (TVAP), nVAP and sVAP on cell proliferation and wound healing.

Materials and methods

Polypeptides and reagents

TVAP was isolated from Wapiti (*Cervus elaphus* Linnaeus) velvet antler in Changchun College of Traditional Chinese Medicine (protein content 82.3%, nVAP accounts for 60%). It was slightly yellow-coloured powder and could be readily dissolved in the water. nVAP was further isolated from TVAP. Purity of TVAP was about 99.4%. It was white-coloured fluffy powder. sVAP was synthesized based on the sequence of nVAP.

NIH 3T3 (Swiss rat fibroblast) cells was bought from Changchun Institute of Biological Material Manufacture. [^{3}H]-TdR was bought from Changchun College of Atomic Energy. Dispase and trypsin were bought from Sigma.

Instrument

WALLAC 1409 DSA Scintilitant Counter, Perkin Elmer. Accuracy: 0.01.

Animals

Wistar rat: body weight, 170-200 g; female and male were 4-5 week-old; newborn was within 24 hr.

New Zealand white rabbit: body weight, 400-700 g.

These animals were bought from Norman Bethune Medical University in Changchun.

Skin wound model

Wistar rats were anaesthetised using ether. The hair of the wounding area on each rat back was thoroughly shaved and sterilised using iodine tincture. A round disc (1.5 cm in diameter and 1.78 cm in area) of full thickness skin was removed using a specifically designed punch. The day on which the wound was created was assigned day 0. The base of Ointment was made of sheep wool lotion, Vaseline and water. Subsequently, 16 and 4 mg TVAP was added into 5 g base respectively. Two different concentrations of the ointment were produced containing the final concentration of 3.2 mg/g and 0.8 mg/g. The ointment was then applied topically on the wound on every other day at the dose of 50 mg/time/rat within 20 days. Only the ointment base was used for the control group. The diameter of each wound was measured and the area was calculated out. The area of each wound at day 0 was assigned 100%. Wound healing rate = the remaining wound area / the wound area on day 0 x 100%. Animals were killed on day 22 and the wound tissue was collected for histological evaluation.

Isolation and culture of epidermal cells

The dorsal skin was collected from new born Wistar rats. The subcutaneous tissue was trimmed off from the sampled skin. The sampled skin was cut into small pieces around 0.5 cm x 0.5 cm. The small pieces were washed in Hanks containing penicillin at 100 u/mL for 1 hr, and transferred into 10 ml digestive solution containing dispase at 1.25 u/mL at 4°C for 20 – 22 hr. Epidermis and dermis were then separated. The isolated epidermal tissue was further digested using 0.25% trypsin at 37°C for 15 min, and pipetted up and down by adding MEM medium to disaggregate epidermal cells. The digests were then filtered through 45 μm pore size nylon membrane. The filter-throughs were counted and cultured in culture medium.

Isolation and culture of chondrocytes

Ribs of new born NZ white rabbits (4 – 5 weeks old) were aseptically collected. Rest and hypertrophic zones were separated and cut into small pieces (1-3 mm^2/piece) respectively. The small pieces were washed twice in PBS and digested in 0.1% EDTA and 0.25% trypsin at 37°C for 1 hr, and then rinsed in PBS. The digests were further digested in 0.2% collagenase II at 37°C for 3 hr and subsequently filtered through 45 μm pore size nylon membrane. The filter-throughs were centrifuged at 1500 rpm for 5 min to collect the disaggregated cells. The cells were cultured in DMEM medium containing 10% FBS after counting the numbers.

$[H^3]$-TdR incorporation into DNA

Isolated epidermal cells were seeded in 96-well plates (0.1 ml medium/well) at the density of 2 x 10^5 cells/ml and cultured in a CO2 incubator. 0.1 ml MEM medium containing different concentrations of samples was added into each well after 24 hr incubation. $[H^3]$-TdR (10 μl containing 37 kBq/well) was added into each well 3 hr before the termination of incubation. After the termination of incubation, the medium of each well was removed by aspiration, and 0.1 ml of 0.25% trypsin was added into each well and incubated further for 30 min. The cells were detached by pepitting up and down, and equal volume of 10% TCA was added into each well at 4° C overnight. Cells were transferred onto 49 type of glass fibre filter paper, which was then dried at 60°C and transferred into a vial containing 3 ml scintillitant. The radio activity was measured using a β-counter.

Cartilaginous cells were seeded in 96-well plates (0.1 ml medium/well) at the density of 1 x 10^5/ml and cultured for 6 days. The medium was replaced by serum-free DMEM containing predetermined concentration of samples and further cultured for 24 hr. $[H^3]$-TdR (10 μl containing 37 kBq/well) was added into each well 3 hr before the termination of incubation. After removal of medium from each well, the cells were washed for 3 times using cold PBS, 2 times using cold 5% TCA and 1 time using mixture of ethanol-ether (3:1). The cells were dissolved in 0.1 ml of 0.5 mol NaOH overnight, and then 10 μl of 6 mol HCL. The solution was transferred to filter paper. After

drying at 60^0C for 5 hr, the paper was transferred into a vial containing 3 ml scintillitant. The radio activity was measured using a β-counter.

NIH 3T3 cell line was passaged in IMDM medium containing 7 – 8% new born calf serum at 37^0C. The cells were then seeded in 96-well plates (0.1 ml medium/well) at the density of 5 x 10^3/ml and cultured for 24 hr. The medium was then replaced using 0.1 ml serum-free medium containing different concentrations of dissolved samples for further 24 hr incubation. [H^3]-TdR (10 µl containing 37 kBq/well) was added into each well 4 hr before the termination of incubation. The rest of steps were the same to epidermal cell culture.

Statistics

Student test was used to analyse the results, which were expressed as $\bar{x} \pm s$.

Results

1. Effects of TVAP on rat skin wound healing

Ointment containing 0.8 or 3.2 mg/g of TVAP significantly promoted rat skin wound healing. In the higher dosage group, on day 7 the wound area was significantly smaller than that of the control. In the lower dosage group, the wound had essentially healed on day 14 (Table 1). Pathological observation on day 22 showed that in the control group, wound healing quality was poor. In some cases, reepithelialization did not occur over the wound region, and in others the wound area was only partially covered by epidermis. This partially healed epidermis was thin and discontinuous. The dermis tissue was full of disorganised blood vessels (Figure 1). However, in the higher dosage group (3.2 mg/g), the wound area was fully covered with epidermis, which was thick and more akin to the normal skin (Figure 2). In the lower dosage group (0.8 mg/g), the wound area was covered with full epidermis, but the epidermis layer was relatively thin and the dermis contained some disorganised blood vessels (Figure 3). Therefore, these results demonstrated that TVAP could not only speed up the wound healing process, but also increased the wound healing quality.

2. Mitogenic effects of TVAP or nVAP on epidermal cells, cartilage cells and fibroblasts

Both TVAP and nVAP promoted [H^3]-TdR incorporating into primary cultured epidermal cell DNA. The effective dosage on promoting proliferation of epidermal cells for nVAP was 0.4 mg/L, but for TVAP was 5 mg/L. This manifests that the biological active substances on epidermal cell proliferation and on wound healing were mainly in nVAP. nVAP at 10 – 50 mg/L and TVAP at 25 – 50 mg/L also promoted proliferation of cartilage cells. nVAP at 1 – 40 mg/L stimulated proliferation of the fibroblasts collected from mouse embryos (Table 2).

3. Mitogenic effects of sVAP on epidermal cells and fibroblasts

sVAP at 5 – 40 mg/L promoted proliferation of rat epithelial cells and mouse fibroblasts (Table 3).

Discussion

When studying bioactive polypeptides from wapiti velvet, we found that these polypeptides can speed up variety kinds of wound healing, particularly have strong mitogenic effects on epidermal cells and fibroblasts. Because velvet extracts per se do not have effects of anti-inflammation and anti-bacteria, we postulate that its effects on wound healing wound be realised through promoting proliferation and differentiation of the cells from the wound region. In order to identify the true bioactive substance from velvet antler, we established a means of measurement of the incorporation rate of [^{3}H]-TdR into epidermal cells, based on which we isolated a monomer from TVAP, which was called nVAP. The purity of nVAP was about 99.4%, and nVAP has mitogenic effects on epidermal cells in a dose-dependent manner within the range of nanogram (400ng/L). Due to the low recover rate of nVAP from velvet antler (1.5×10^{-3}%), it cannot be used for the testing on the treatment of rat skin wound, we used TVAP containing 60% of nVAP for the in vivo trial. We obtained ideal results. Therefore, we think nVAP is the true bioactive component of TVAP for promoting wound healing. Based on the sequence of nVAP, we synthesized this peptide, called sVAP. sVAP has similar mitogenic effects on epidermal cells and dermal cells. The only difference between nVAP and sVAP is that the molecular weight of sVAP is 16 u smaller than that of nVAP.

In a previous study, we reported that both TVAP and the B component (MW: 5,788 u) of TVAP from sika deer velvet antlers have mitogenic effects on bone and cartilaginous cells, and the effects of promoting bone fracture repair. In the present study, we found that both TVAP and nVAP from wapiti velvet antler have mitogenic effects on epidermal cells and the effects of promoting skin wound healing. Therefore, TVAP from different deer species may have different composition and different biological activity. In order to confirm this, we did following analyses. Firstly, using the same techniques, polypeptides were extracted from both wapiti and sika deer velvet antler, and then were systemically analysed using techniques of HPLC, MALDI-TOF MS and SDS-PAGE. The results showed that there were significant differences in physical, chemical and biological properties between the polypeptides of sika velvet antler and wapiti velvet antler. Based on the aforementioned properties, we produced injectable lyophilised powders (second class TCM drug) for the treatment of bone fracture (sika deer velvet polypeptides) and for the treatment of skin wound (wapiti velvet polypeptides).

One of the important events for wound healing is cell proliferation and differentiation. When the skin wound area is relatively big, the healing starts from the proliferation of epidermal cells, and then the propagation of fibroblasts. The final outcome of adult wound healing is scar formation. That velvet polypeptides can promote wound healing is because they can stimulate the proliferation of epidermal cells and fibroblasts. Currently we are studying the effects of velvet polypeptides on

the proliferation and differentiation of endothelial cells and nerve cells, as well as on synthesis and degradation of extracellular matrix.